VERIFICATION OF TRANSLATION

Re: JAPANESE PATENT APPLICATION NO. 1999-167736

I, Hiromichi KAKEHI, of Kitahama TNK Building, 7-1, Dosho-machi 1-chome, Chuo-ku, Osaka-shi, Osaka 541-0045, Japan hereby declare that I am the translator of the document attached and certify that the following is true translation to the best of my knowledge and belief.

Signature of translator H. Kakehi

Hiromichi KAKEHI

Dated this 6th day of October, 2004

[Document Name] Specification

[Title of the Invention] Method for Determining HIV-1 Subtype

[Claims]

[Claim 1] A method for determining HIV-1 subtypes, which comprises the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5′ terminal and 3′ terminal nucleotide sequences is different depending on the HIV-1 subtype, and detecting the subtype depending on whether or not the nucleic acid has been amplified.

[Claim 2] The method according to Claim 1, wherein the target sequence is 100 to 2500 nucleotides long.

[Claim 3] The method according to Claim 1 or Claim 2, wherein the sequence from the 1st through 30th bases from the 3′ terminal and/or 5′ terminal of the target sequence is different depending on the subtype.

[Claim 4] The method according to Claim 3, wherein the 3′ terminal of the target sequence is in the C3 region of the env gene of HIV-1.

[Claim 5] The method according to Claim 4, wherein the 5′ terminal of the target sequence is in the C2 region of the env gene of HIV-1.

[Claim 6] The method according to any of Claims 1 through 5, wherein different amplification reactions are carried out using different primer pairs, to detect different subtypes.

[Claim 7] The method according to Claim 6, wherein at least two different subtypes are detected by carrying out amplification reactions at least twice with different primer pairs consisting of a primer (primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1.

[Claim 8] The method according to any of Claims 1 through 7, wherein the first amplification reaction is carried out with the first primer pair using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, and then the second amplification reaction is carried out with the second primer pair using as a target sequence a portion of said target sequence wherein at least one of the 5′ terminal and 3′ terminal nucleotide sequences is different depending on the subtype, to detect the subtype depending on whether or not the

nucleic acid has been amplified by the second amplification reaction.

[Claim 9] The method according to Claim 8, wherein the second primer pair consists of a primer (primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1; and the first primer pair consists of a primer (primer 3) that includes a sequence complementary to a portion of a nucleotide sequence (nucleotide sequence 3) of the outside region of the 3' terminal of nucleotide sequence 1 of the env gene of HIV-1, and a primer (primer 4) that includes a sequence complementary to a portion of a nucleotide sequence (nucleotide sequence 4) of the outside region of the 5' terminal of nucleotide sequence 2 of the env gene of HIV-1.

[Claim 10] The method according to Claim 8 or Claim 9, wherein at least two subtypes are distinguished by repeating at least once, with different second primer pairs, a series of operations comprising: the first amplification reaction that is carried out with the first primer pair using as a target sequence a portion of a

nucleotide sequence of the env gene of HIV-1; the second amplification reaction that is then carried out with the second primer pair using as a target sequence a nucleotide sequence within said target sequence; to detect subtypes depending on whether or not the nucleic acid has been amplified by the second amplification reaction.

[Claim 11] The method according to Claim 10, wherein subtypes A, B, and E are distinguished by:

(a) detecting subtype A using as the first primer pair primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and using as the second primer pair, primer 11QA containing nucleotide sequence CTCCTGAGGGGTTAGCAAAG(Sequence ID No. 1) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG(Sequence ID No. 4);

(b) detecting subtype B using as the first primer pair primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC(Sequence ID No. 6) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG(Sequence ID No. 9), and using as the second primer pair primer 11BB containing nucleotide sequence CTGTGCATTACAATTTCTGG(Sequence ID No. 2) and primer 10 containing nucleotide sequence

AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4); and

(c) detecting subtype E using as the first primer pair primer 12E containing nucleotide sequence GCAATAGAAAAATTCCCCTC(Sequence ID No. 7) and primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG(Sequence ID No. 8), and using as the second primer pair primer 11QE containing nucleotide sequence CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4).

[Claim 12] The method according to any of Claims 1 through 11, which further comprises the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the HIV-1 genome, the nucleotide sequence being highly conserved among all subtypes, and ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified.

[Claim 13] A kit for determining HIV-1 subtypes, which comprises a primer pair in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5′ terminal and 3′ terminal nucleotides is different depending on the subtype.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to a method for determining HIV-1 subtypes, and a kit for determining HIV-1 subtypes.

[0002]

[Prior Art]

The human immunodeficiency virus (hereinafter, referred to as "HIV") is a virus causing acquired immune deficiency syndrome (hereinafter, referred to as "AIDS"), and type 1 (HIV-1) and type 2 (HIV-2) are known. Most cases involve HIV-1, for which various subtypes have been discovered.

Determining the HIV-1 subtype in infected individuals is important for assessing the reliability of virological test results (particularly the drug resistance based on genotype or the determination of plasma HIV-1 RNA concentration) and the route of infection. HIV-1 subtypes are generally determined through the sequencing of specific regions of the virus genome and phylogenetic analysis of the results, but these are complicated and expensive procedures.

[0003]

[Problem to be Solved by the Invention]

Thus, an object of the present invention is to

provide a simpler method for determining HIV-1 subtypes.

Another object of the present invention is to provide a kit for determining HIV-1 subtypes.

[0004]

[Means for Solving the Problem]

The inventors have designed various subtype-specific primers and have successfully used them to amplify nucleic acid in samples for rapid determination of HIV-1 subtypes thereby to complete the present invention.

Specifically, the present invention provides a method for determining HIV-1 subtypes, which comprises the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5′ terminal and 3′ terminal nucleotide sequences is different depending on the HIV-1 subtype, and detecting the subtype depending on whether or not the nucleic acid has been amplified. The target sequence should be 100 to 2500 base pairs in length, and preferably 150 to 500 base pairs in length. In the above method, the sequence from the 1st through 30th bases from the 3′ terminal and/or 5′ terminal of the target sequence should be different depending on the subtype. For example, the 3′ terminal of the target sequence may be in the C3 region of the env gene of HIV-1. The 5′ terminal of the target sequence may be in the C2 region of

the env gene of HIV-1. Different subtypes can be detected by different amplification reactions using different primer pairs. For example, at least two subtypes can be detected by carrying out amplification reactions at least twice with different primer pairs consisting of a primer (primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1.

[0005]

The first amplification reaction may be carried out with the first primer pair using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, the second amplification reaction may then be carried out with the second primer pair using as a target sequence a portion of the aforementioned target sequence wherein at least one of the 5′ terminal and 3′ terminal nucleotide sequences is different depending on the subtype, and the subtype may be detected depending on whether or not the nucleic acid has been amplified by the second amplification reaction. For example, the second primer pair consists of a primer (primer 1) that includes a

sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) in the C2 region of the env gene of HIV-1; and the first primer pair may consist of a primer (primer 3) that includes a sequence complementary to a portion of a nucleotide sequence (nucleotide sequence 3) of the outside region of the 3′ terminal of nucleotide sequence 1 of the env gene of HIV-1, and a primer (primer 4) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 4) of the outside region of the 5′ terminal of nucleotide sequence 2 of the env gene of HIV-1. At least two subtypes can be distinguished by repeating at least once the following series of operations with different second primer pairs, the operations comprising the first amplification reaction that is carried out with the first primer pair using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, the second amplification reaction that is then carried out with the second primer pair using as a target sequence a nucleotide sequence within the above target sequence, and the detection of subtypes depending on whether or not the nucleic acid has been amplified by

the second amplification reaction. For example, subtypes A, B, and E can be distinguished by: (a) detecting subtype A using as the first primer pair primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and using as the second primer pair primer 11QA containing nucleotide sequence CTCCTGAGGGGTTAGCAAAG (Sequence ID No. 1) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4);

(b) detecting subtype B using as the first primer pair primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC(Sequence ID No. 6) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11BB containing nucleotide sequence CTGTGCATTACAATTTCTGG (Sequence ID No. 2) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4); and

(c) detecting subtype E using as the first primer pair primer 12E containing nucleotide sequence GCAATAGAAAAATTCCCCTC (Sequence ID No. 7) and primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and using as the second primer pair primer 11QE containing nucleotide sequence

CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4).

[0006]

The method of the present invention may further comprise the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the HIV-1 genome, the nucleotide sequence being highly conserved among all subtypes and ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified.

Another object of the present invention is to provide a kit for determining HIV-1 subtypes, comprising a primer pair in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5′ terminal and 3′ terminal nucleotide sequences is different depending on the subtype.

[0007]

[Mode for Carrying out the Invention]

Hereinafter, embodiments of the present invention are illustrated below.

A sample of blood, lymph, spinal fluid, semen, lymph node, or the like is taken from individuals suspected of HIV-1 infection, infected individuals and patients confirmed with HIV-1 infection, patients being treated for

HIV-1, and the like. DNA is extracted using a QIAamp Blood Kit (QIAGEN), either directly or after monocytes have been isolated from the sample by Ficoll-Paque density gradient centrifugation (Pharmacia). Alternatively, RNA is extracted using a QIAamp Viral RNA Kit (QIAGEN) from plasma. The DNA or RNA concentration is then determined based on the absorption at the wavelength of 260 nm.

[0008]

The nucleic acid is then treated in PCR, and preferably nested PCR.

The use of nested PCR is described below. Nested PCR involves designing the second primer pair inside a target sequence amplified with another primer pair (first primer pair), carrying out the first PCR step, and then diluting the reaction product as a new template for the second PCR step, and then carrying out the second PCR step. Undesirable sequences are sometimes amplified in addition to the target sequence in the first PCR step. However, there is very little possibility that undesirable fragments amplified during the first PCR step have a sequence with which the primers of the second primer pair will anneal. The second PCR step is thus carried out for selectively amplifying the target sequence.

[0009]

The initial PCR step (first PCR) is first carried

out using different primer pairs specific to each subtype to be distinguished (such as subtype A, subtype B, and subtype E). Alternatively, universal primer pairs allowing any type of subtypes to be amplified can be used instead of subtype-specific primer pairs.

An example of a subtype-specific primer pair is a primer pair consisting of a primer (primer 4') which includes a sequence complementary to a portion of a nucleotide sequence in the C2 region of the env gene of HIV-1 and a primer (primer 3') which includes a sequence complementary to a portion of the nucleotide sequence of the C3 region of the env gene for HIV-1 that differs depending on subtype (that is, subtype-specific nucleotide sequence). Since the C2 region of the env gene of HIV-1 has a nucleotide sequence that differs depending on the subtype, as shown in Figure 1, the nucleotide sequence may be selected from this region to design primer 4'. Because the C3 region of the env gene of HIV-1 varies depending on the subtype, as shown in Figure 2, the nucleotide sequence may be selected from this region to design primer 3'. The primer should generally be 18 to 30 base pairs, and preferably 20 to 25 base pairs in length. Specifically, the following primers can be used.

[0010]

A subtype A-specific primer pair for the first PCR

and their nucleotide sequences

9AE/12A

primer 9AE: CACAGTACAATGCACACATG (Sequence ID No. 8)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

[0011]

A subtype B-specific primer pair for the first PCR and their nucleotide sequences

9B/12B

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12B: ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6)

[0012]

A subtype E-specific primer pair for the first PCR and their nucleotide sequences

9AE/12E

primer 9AE: CACAGTACAATGCACACATG (Sequence ID No. 8)

primer 12E: GCAATAGAAAAATTCCCCTC (Sequence ID No. 7)

[0013]

A subtype C-specific primer pair for the first PCR and their nucleotide sequences

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

[0014]

A subtype D-specific primer pair for the first PCR and their nucleotide sequences

9B/12B

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12B: ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6)

[0015]

A subtype F-specific primer pair for the first PCR and their nucleotide sequences

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

[0016]

A subtype G-specific primer pair for the first PCR and their nucleotide sequences

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

[0017]

A subtype H-specific primer pair for the first PCR and their nucleotide sequences

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

[0018]

Any subtype may be amplified by using a mixture of the above-mentioned subtype-specific primers instead of a single use of the above subtype-specific primer pair.

1/1000 to 1/5 (for example, 1/50) of the PCR products is used to carry out the next PCR (second PCR) with another primer pair specific to each subtype. The primer pair for the second PCR is designed within the target sequence amplified during the first PCR. For example, at least one of the primers forming the subtype-specific primer pair for the second PCR can be a primer (primer 1) containing a sequence complementary to a portion of the subtype-specific nucleotide sequence of the C2 region of the env gene for HIV-1. Since the nucleotide sequence of the C2 region of the env gene for HIV-1 differs depending on subtype, as shown in Figure 1, a nucleotide sequence from this region can be selected to design the primer. Figure 2 gives the nucleotide sequence of the 3' adjacent region (C3 region) of the V3 region of the env gene for various subtypes of HIV-1. Since the nucleotide sequence varies depending on the subtype, a suitable sequence can be selected to design a primer. To design a subtype-specific primer, phylogenetic analysis is employed to select nucleotide sequences of a given subtype that are as genetically remote as possible from the corresponding nucleotide sequences of other subtypes. An example can include a primer (primer 2) containing a sequence complementary to a portion of the nucleotide sequence of the C3 region of the

env gene for HIV-1. Specifically, the primer pair containing the following nucleotide sequences can be used.

[0019]

A subtype A-specific primer pair for the second PCR and their nucleotide sequences

10/11QA

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11QA: CTCCTGAGGGGTTAGCAAAG(Sequence ID No. 1)

[0020]

A subtype B-specific primer pair for the second PCR and their nucleotide sequences

10/11BB

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11BB: CTGTGCATTACAATTTCTGG (Sequence ID No. 2)

[0021]

A subtype E-specific primer pair for the second PCR and their nucleotide sequences

10/11QE

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11QE: CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3)

[0022]

A subtype C-specific primer pair for the second PCR

10C/11RC

primer 10C: AAATGGTAGCCTAGCAGAAG (Sequence ID No. 10)

primer 11RC: CTCCTGAGGATGGTGCAAATTT (Sequence ID No. 13)

[0023]

A subtype D-specific primer pair for the second PCR 10/11RD

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11RD: CTCCTGAGGATGGTTTAAAAAT (Sequence ID No. 14)

[0024]

A subtype F-specific primer pair for the second PCR 10C/11RF

primer 10C: AAATGGTAGCCTAGCAGAAG (Sequence ID No. 10)

primer 11RF: CTCCTGAGGATGAGTTAAATTT (Sequence ID No. 15)

[0025]

A subtype G-specific primer pair for the second PCR 10G/11SG

primer 10G: GAATGGCAGTTTAGCAGAAG (Sequence ID No. 11)

primer 11SG: TCCTGCAGATGAGTTAAAGG (Sequence ID No. 16)

[0026]

A subtype H-specific primer pair for the second PCR 10H/11SH

primer 10H: GTCAAATGGCAGTTTAGCAG (Sequence ID No. 12)

primer 11SH: TCCTGAGGATGGTTTAAAGG (Sequence ID No. 17)

[0027]

A subtype universal primer pair for the second PCR 10/11LB

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11LB: AATTTCTGGGTCCCCTCCTG (Sequence ID No. 18)

[0028]

A subtype universal primer pair for the second PCR 10/11LE

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11LE: AATTTCTAGATCTCCTCCTG (Sequence ID No. 19)

[0029]

The PCR procedures and reaction conditions may be in accordance with those in Bruisten S. et al., *AIDS Res Hum Retroviruses* 1993, 9:259-265, but the hot start method is preferred. In hot start PCR, the PCR reaction solution is kept on a hot plate for start up at an elevated temperature (usually 90°C or higher).

However, it sometimes happens that no subtype is detected in an attempt to determine the HIV-1 subtype in such a method. Possible causes may be that the HIV-1 DNA concentration is below the detection threshold, or the presence of numerous variants at the primer binding site.

[0030]

To deal with the former possibility, the above method can be implemented after extracting RNA from plasma and converting the RNA into cDNA using reverse transcriptase, since the concentration of HIV-1 is generally higher in plasma than in cells.

To deal with the latter possibility, the determination of the subtype by the method of the present

invention is held off, another genetic region of HIV-1 is amplified by PCR to determine the nucleotide sequence, and the subtype is determined by a conventional method (Note: HIV-1 infection is generally diagnosed by detecting antibodies. This invention is not a method for diagnosing HIV-1 infection.).

The PCR reaction products are separated by agarose gel electrophoresis and detected by ethidium bromide staining. Although distinct bands can be observed with the use of primers consistent with the subtype of the HIV-1 in sample DNA, the bands are indistinct or not observed at all when the primers are not consistent with the subtype of the HIV-1 in sample DNA. The HIV-1 subtype is determined in this way.

[0031]

The present invention also encompasses a kit for determining HIV-1 subtypes, comprising a primer pair in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5′ terminal and 3′ terminal nucleotide sequences is different depending on the subtype. Examples of primer pairs include primer pairs (inner primers) for the second PCR such as those above, and combinations of primer pairs for the first PCR (outer primers) and primer pairs for the second PCR. The kit of the present invention may also

include dNTP mixtures, reaction buffers, DNA polymerase. To minimize the effects caused by inconsistencies between the primer and analyte HIV-1 DNA base pairs, the magnesium ion concentration in the reaction buffer should be increased from the usual concentration of 1.5 mM to 4 mM.

The components constituting the diagnostic kit may be packaged individually, assembled, or bundled in containers such as vials and tubes, and further the containers may be in supporting means divided for housing such components.

[0032]

[Examples]

The present invention is illustrated in detail with the following examples, but the scope of the present invention is not limited to these examples.

[Example 1]

Subjects and Method

1) Subtype-specific specimens to study method for determining subtype

Specimens were prepared by extracting DNA from the blood of 3 HIV-infected subjects determined to be subtype A, 8 HIV-infected subjects determined to be subtype B and 3 HIV-infected subjects determined to be subtype E, through env gene sequencing and phylogenetic analysis.

[0033]

2) Subjects for determining subtype

The HIV-1 subtype was determined using 8 HIV-infected subjects who either visited or were hospitalized in hospitals in Tokyo.

3) Preparation of DNA from blood of HIV patients

10 ml-peripheral blood was collected from the above HIV-infected patients. Sodium citrate was used as an anticoagulant. Monocytes were separated from the peripheral blood by Ficoll-Paque (Pharmacia) density gradient centrifugation, and DNA was then prepared using a QIAamp Blood Kit (QIAGEN). The DNA was dissolved in pure water or buffer containing 1 mM EDTA, and was stored at -20°C until immediately before use. 0.5 μg DNA was used in PCR.

[0034]

4) Detection of subtypes A, B, and E by PCR

Figure 3 gives the nucleotide sequences of the primers used in PCR.

For subtype A-specific detection, nested PCR was carried out using 9AE and 12A as the primers for the first PCR, and 10 and 11QA as the primers for the second PCR. For subtype B-specific detection, nested PCR was carried out using 9B and 12B as the primers for the first PCR, and 10 and 11BB as the primers for the second PCR. For subtype E-specific detection, nested PCR was carried out

using 9AE and 12E as the primers for the first PCR, and 10 and 11QE as the primers for the second PCR (Figure 3).

[0035]

PCR was carried out for 30 cycles, wherein one cycle consisted of 15 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C, with 100 μL reaction solution (10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dNTP, 1.0 μM primer, 2.5 units Taq polymerase) using 0.5 μg sample DNA prepared from HIV-infected subjects. Using 2 μL reaction solution obtained from the first PCR, the second PCR was carried out for 25 cycles under the same conditions with the exception of using 30 seconds at 60°C instead of 30 seconds at 56°C.

PCR products (subtypes A and E: 317 bp; subtype B: 342 bp) were detected by isolation through electrophoresis with 2% agarose gel, and by subsequent ethidium bromide staining.

[0036]

Results

1) Study of subtype determination by PCR of subtype-specific specimens

The following results were obtained for specimens whose subtypes had been determined by virus genome sequencing. Only subtype A specimens were positive, while subtype B and E specimens were all negative, in PCR using

primers for the detection of subtype A. Only subtype B specimens were positive, while subtype A and E specimens were all negative, in PCR using primers for the detection of subtype B. Only subtype E specimens were positive, while subtype A and B specimens were all negative, in PCR using primers for the detection of subtype E (Figure 4).

[0037]

2) Determination of subtype of HIV patients by PCR

Table 1 gives the results obtained in the determination of HIV-1 subtypes in 8 HIV-infected patients who either visited or were hospitalized in hospitals in Tokyo.

[0038]

Table 1 The results obtained in the determination of unknown subtypes in 8 specimens

Case	primer pair for subtype A	primer pair for subtype B	primer pair for subtype E
P18	−	+	−
P19	−	+	−
P20	−	+	−
P21	−	−	+
P22	−	?	−
P23	−	−	+
P24	−	+	−
P25	−	+	−

In the table above, + denotes detection of HIV-1 specific DNA bands, - denotes non-detection thereof. The symbol "?" for Case 22 denotes detection of shorter bands than expectation.

[0039]

Based on these results, Cases P18, P19, P20, P24, and P25 were diagnosed as being infected with subtype B, and Cases P21 and P23 were diagnosed as being infected with subtype E. Although a DNA band was detected only with the use of a primer pair for subtype B in Case P22, it was shorter than expected, so determination was postponed. To verify that the above results were correct, the amplified DNA was sequenced and phylogenetically analyzed, showing that the results of the phylogenetic analysis were consistent with those in Table 1. Case P22, which was postponed, turned out to be subtype B.

[0040]

The results in 1) and 2) demonstrate that this method was able to correctly diagnose the subtype in 21 out of 22 cases. The determination was postponed in the remaining one case. That is, the present method has been shown to be a simple and reliable method for determining subtypes.

The method of the present invention allows the determination of HIV-1 subtype at a cost of about ¥2,000 per specimen. The time needed to determine the subtype in treating all 8 specimens at once was 2 hours for the isolation of DNA, 6 hours for PCR, and about 1 hour for electrophoresis.

[0041]

[Effect of the Invention]

The present invention provides a simple method for determining HIV-1 subtypes. The invention also provides an effective means for determining HIV-1 subtypes.

[0042]

[Sequence Listing]

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<213> Artificial Sequence

<220>

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 15

ctcctgagga tgagttaaat tt 22

<210> 16

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 16

tcctgcagat gagttaaagg 20

<210> 17

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 17

tcctgaggat ggtttaaagg 20

<210> 18

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 18

aatttctggg tcccctcctg 20

<210> 19

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 19

aatttctaga tctcctcctg 20

[0043]

[Free Text of Sequence Listing]

Sequence ID No. 1 gives the nucleotide sequence for primer 11QA.

Sequence ID No. 2 gives the nucleotide sequence for

primer 11BB.

Sequence ID No. 3 gives the nucleotide sequence for primer 11QE.

Sequence ID No. 4 gives the nucleotide sequence for primer 10.

Sequence ID No. 5 gives the nucleotide sequence for primer 12A.

[0044]

Sequence ID No. 6 gives the nucleotide sequence for primer 12B.

Sequence ID No. 7 gives the nucleotide sequence for primer 12E.

Sequence ID No. 8 gives the nucleotide sequence for primer 9AE.

Sequence ID No. 9 gives the nucleotide sequence for primer 9B.

Sequence ID No. 10 gives the nucleotide sequence for primer 10C.

[0045]

Sequence ID No. 11 gives the nucleotide sequence for primer 10G.

Sequence ID No. 12 gives the nucleotide sequence for primer 10H.

Sequence ID No. 13 gives the nucleotide sequence for primer 11RC.

Sequence ID No. 14 gives the nucleotide sequence for primer 11RD.

Sequence ID No. 15 gives the nucleotide sequence for primer 11RF.

[0046]

Sequence ID No. 16 gives the nucleotide sequence for primer 11SG.

Sequence ID No. 17 gives the nucleotide sequence for primer 11SH.

Sequence ID No. 18 gives the nucleotide sequence for primer 11LB.

Sequence ID No. 19 gives the nucleotide sequence for primer 11LE.

[Brief Description of the Drawing]

[Figure 1]

Figure 1 illustrates nucleotide sequences of the 5′ adjacent region (C2 region) of the V3 region for the env gene in various subtypes of HIV-1. Capital letters indicate that the nucleotides are entirely the same within a given subtype, and lower case letters indicate the presence of nucleotide variants that are different within a given subtype. A question mark "?" indicates that a consensus nucleotide was not determined because of too many variants. A dash "-" indicates a nucleotide identical to that in subtype A. A period "." indicates

the absence of a nucleotide in the corresponding site.

[Figure 2]

Figure 2 illustrates nucleotide sequences of the 3′ adjacent region (C3 region) of the V3 region for the env gene in various subtypes of HIV-1. Capital letters indicate that the nucleotides are entirely the same within a given subtype, and lower case letters indicate the presence of nucleotide variants that are different within a given subtype. A question mark "?" indicates that a consensus nucleotide was not determined because of too many variants. A dash "-" indicates a nucleotide identical to that in subtype A. A period "." indicates the absence of a nucleotide in the corresponding site.

[Figure 3]

Figure 3 illustrates the design of primers used in nested PCR for determining HIV-1 subtypes.

[Figure 4]

Figure 4 gives the results obtained when subtypes were detected by nested PCR for specimens in which the subtypes had been determined by sequencing of the virus genome.

[Document Name] Drawing

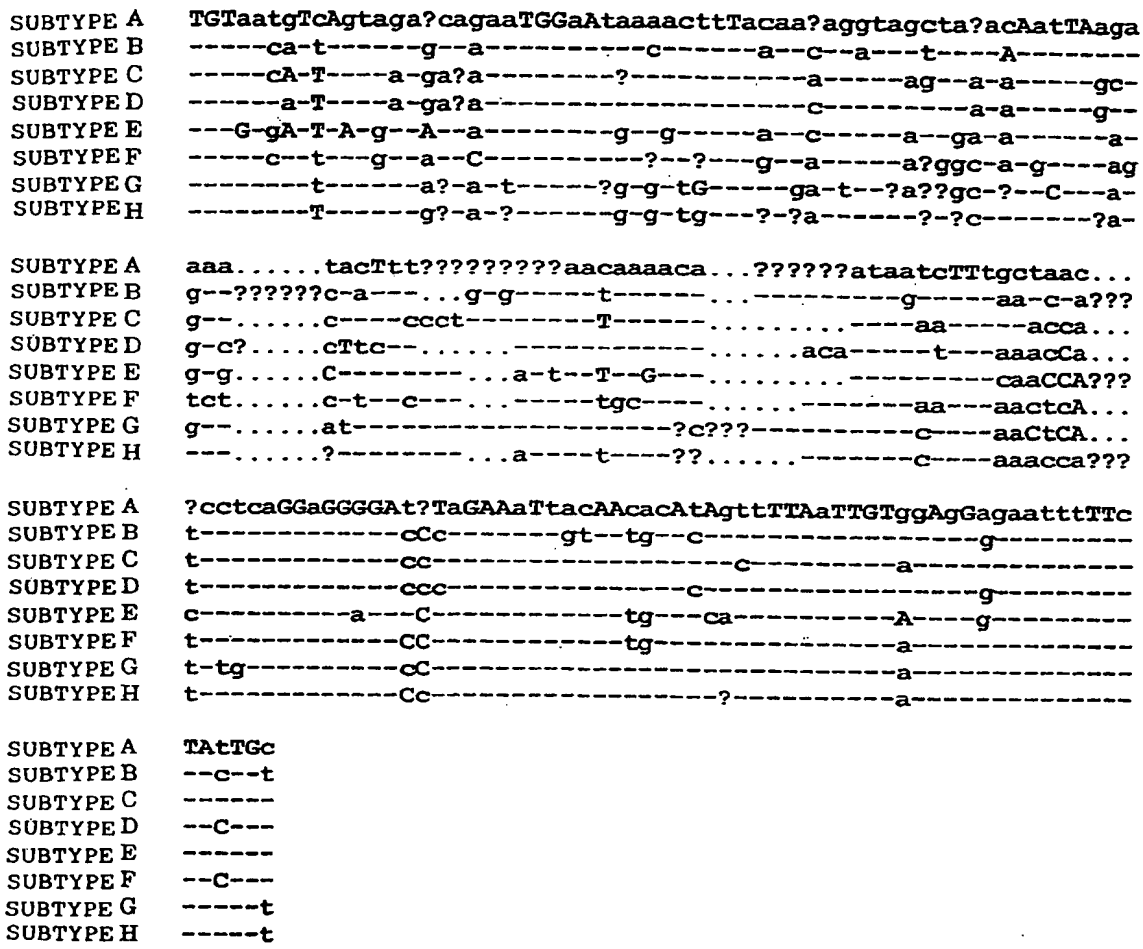
[Figure 1]

The nucleotide sequences of C2 region in subtypes of HIV-1

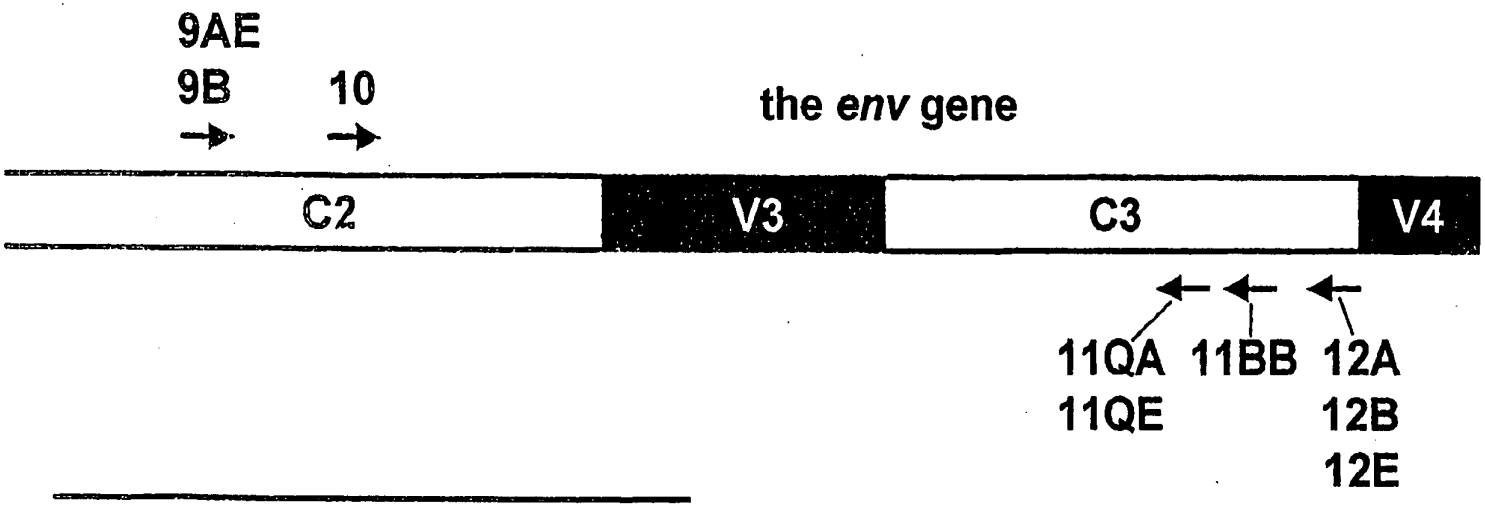
```
SUBTYPE A   TGTAataccTCAgccatTAcAcAgGCtTGtCCaAAggTatCCTTTGAgCCaATTCCCATA
SUBTYPE B   -----c-------t----------c-------------------------------
SUBTYPE C   ----------------A-----a--C----------c--t-----c--------T---
SUBTYPE D   ------------------------g----------a--------------------
SUBTYPE E   --------T----T-----aG--------------a----------T--------t---
SUBTYPE F   ----------A-----------------------T-GG--T-----------
SUBTYPE G   ------gt----A------A-----------------ga?T-----c-----------
SUBTYPE H   ------GT----------A-----------------GAGT-----T-----------

SUBTYPE A   caTTATTGtgCcCCaGCTGGtTttGCgATtCTAAagTGtAa?gataaggagTTcaatGGA
SUBTYPE B   -------------g-------------------------t------a----------
SUBTYPE C   ----------t----------a-----------------ta-----aca--------g
SUBTYPE D   --------------------a-----a--------a-----------A----------g
SUBTYPE E   ----------a-t---------a-------t----------T------a-t--------g
SUBTYPE F   ----------T---------A-----------------T-----aA----------G
SUBTYPE G   ----------T-----------------t---------gg------a-?---------
SUBTYPE H   ----------T-------G---------A---------GG------A----------G

SUBTYPE A   acAGGgccatGcaagAATGTcAGcaCaGTaCAATGcACacATGGaATcAagCCAGtagTa
SUBTYPE B   -----a-----t-ca-------------------t----------t-g----------
SUBTYPE C   -----a------c-t-------------------t----------t--------g---
SUBTYPE D   -----?--------a-------------------t--------g--t-g---------g
SUBTYPE E   ----------t--A---------T----------------------T--------G---
SUBTYPE F   --g-------------------------------T----------T--A-----g---
SUBTYPE G   -----A-----T--a--------T----------T----------T--------g---
SUBTYPE H   --G--AA----T--A--------T----------T----------T--------G---

SUBTYPE A   tCAACtCAaCTgcTGtTaAATGGcAGtcTAGCAgaAgaa???gaggTAatgaTtagaTCT
SUBTYPE B   ------------------------------------...------g-a---------
SUBTYPE C   ----------a----------t--c-----------...---a----a---------
SUBTYPE D   ----------?----g-------------------...---a----A---------
SUBTYPE E   ---------t-------------------------...---A----A--c------
SUBTYPE F   ---------T--T-----------C----------...--ta----A--c------
SUBTYPE G   ---------t-a---c-g---------t----------...--aA----a---------
SUBTYPE H   ---------T-A---C-GTCAAATG-CAGTTT--C---------?a----a---------

SUBTYPE A   gAaaataTcacAaAcAATgccaaaAccaTAaTaGTacAgcTtg??aagcctGTAa?aATt
SUBTYPE B   ------t----gg------t-------------------gaa-g-at-----ga----
SUBTYPE C   ------c-g---------t------a----------t---aAtg-at-----ga----
SUBTYPE D   ------c----------t----?---------------AAtG--t-----?c----
SUBTYPE E   ------C-------------------------G--C---AAT--At-----Ga---C
SUBTYPE F   c---------t--g-t-----A----------------?---AATg-At-----ca----
SUBTYPE G   -----c?-----g----------gt---------g------AAt--a?--a--ga----
SUBTYPE H   -----c------g-----a-----gt---------------AAt--a?-----g-----

SUBTYPE A   aatTGT
SUBTYPE B   ------
SUBTYPE C   gtg---
SUBTYPE D   ------
SUBTYPE E   ------
SUBTYPE F   ------
SUBTYPE G   -??---
SUBTYPE H   --?---
```

[Figure 2]

The nucleotide sequences of C3 region in subtypes of HIV-1



[Figure 3]

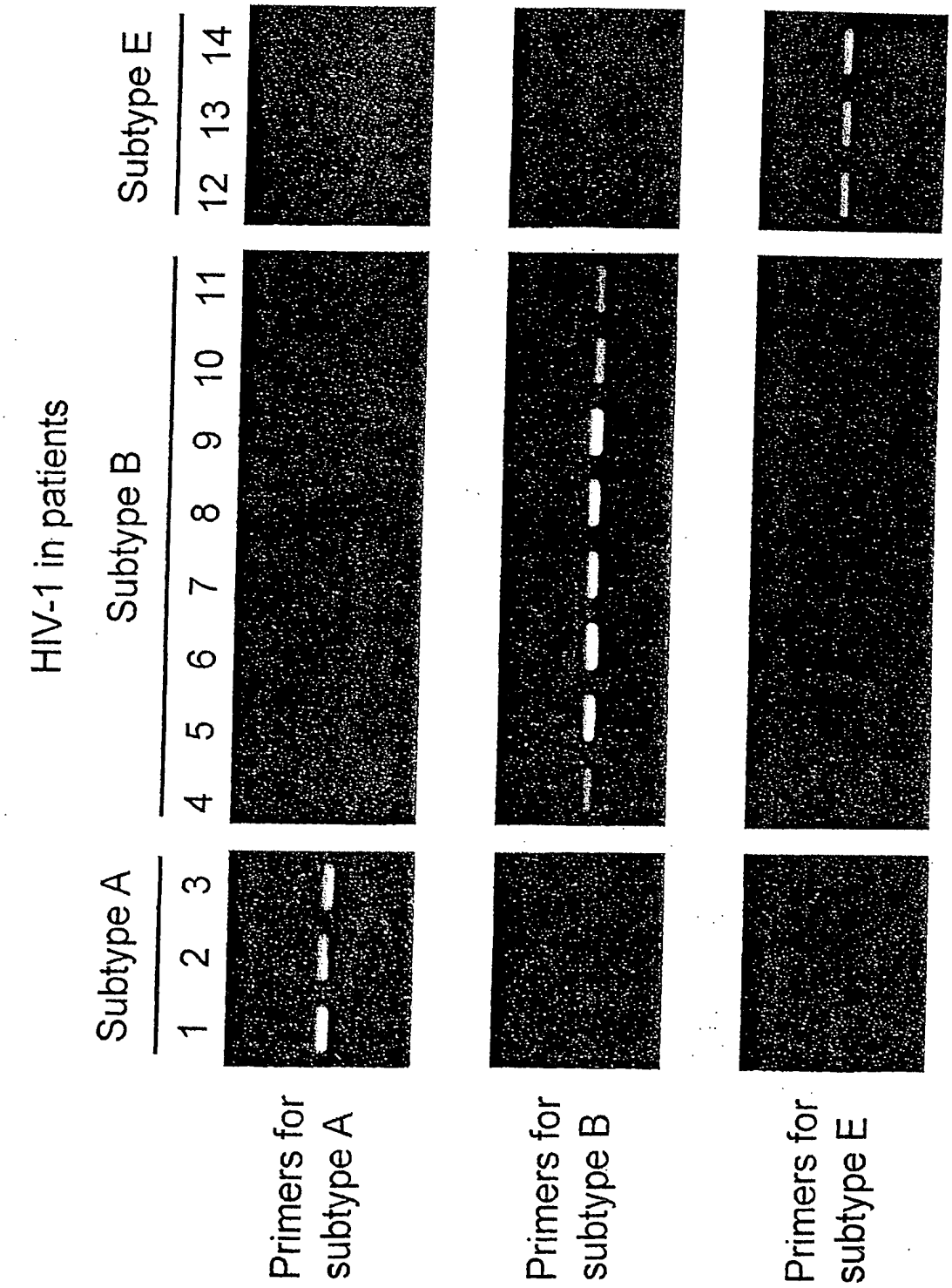


Primers used for		
Subtype	1st PCR	2nd PCR
A	9AE/12A	10/11QA
B	9B/12B	10/11BB
E	9AE/12E	10/11QE

Primers

9AE:	CACAGTACAATGCACACATG
9B:	CACAGTACAATGTACACATG
10:	AAATGGCAGTCTAGCAGAAG
11QA:	CTCCTGAGGGGTTAGCAAAG
11QE:	CTCCTGAGGGTGGTTGAAAG
11BB:	CTGTGCATTACAATTTCTGG
12A:	GCAATAGAAAAATTCTCCTC
12B:	ACAGTAGAAAAATTCCCCTC
12E:	GCAATAGAAAAATTCCCCTC

[Figure 4]


HIV-1 in patients
Subtype A
1 2 3
Subtype B
4 5 6 7 8 9 10 11
Subtype E
12 13 14
Primers for subtype A
Primers for subtype B
Primers for subtype E

[Document Name] Abstract

[Abstract]

[Object] A method for determining HIV-1 subtypes, which comprises the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5′ terminal and 3′ terminal nucleotide sequences is different depending on the HIV-1 subtype, and detecting the subtype depending on whether or not the nucleic acid has been amplified. A kit for determining HIV-1 subtypes, comprising a primer pair in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5′ terminal and 3′ terminal nucleotide sequences is different depending on the subtype.

[Method for Achieving the Object] The present invention provides a simple method for determining HIV-1 subtypes. The present invention also provides an effective method for determining HIV-1 subtypes.

[Selected Figure] None